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Exposure of human astrocytes to leukotriene C_4 promotes a CX3CL1/fractalkine-mediated transmigration of HIV-1-infected $CD4^+$ T cells across an *in vitro* blood–brain barrier model

Jonathan Bertin [a], Pascal Jalaguier [a], Corinne Barat [a], Marc-André Roy [a], Michel J. Tremblay [a,b,*]

[a] Axe des Maladies Infectieuses et Immunitaires, Centre de recherche du Centre Hospitalier Universitaire (CHU) de Québec - pavillon CHUL, Canada
[b] Département de Microbiologie-Infectiologie et Immunologie, Faculté de médecine, Université Laval, Québec, Canada

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ABSTRACT

Eicosanoids, including cysteinylleukotrienes (cysLTs), are found in the central nervous system (CNS) of individuals infected with HIV-1. Few studies have addressed the contribution of cysLTs in HIV-1-associated CNS disorders. We demonstrate that conditioned medium from human astrocytes treated with leukotriene C_4 (LTC_4) increases the transmigration of HIV-1-infected $CD4^+$ T cells across an *in vitro* blood–brain barrier (BBB) model using cultured brain endothelial cells. Additional studies indicate that the higher cell migration is linked with secretion by astrocytes of CX3CL1/fractalkine, a chemokine that has chemoattractant activity for $CD4^+$ T cells. Moreover, we report that the enhanced cell migration across BBB leads to a more important $CD4^+$ T cell-mediated HIV-1 transfer toward macrophages. Altogether data presented in the present study reveal the important role that LTC_4, a metabolite of arachidonic acid, may play in the HIV-1-induced neuroinvasion, neuropathogenesis and disease progression.

Introduction

HIV-1 can invade the central nervous system (CNS) early during the acute phase of the infection (Davis et al., 1992) via virus-infected leukocytes (mostly monocytes and $CD4^+$ T cells) and/or circulating cell-free virions (Kolb et al., 1999; Wu et al., 2000). Once in the CNS, the virus can productively infect several residing cell types such as perivascular macrophages, microglial cells and to some extent astrocytes (Anthony and Bell, 2008; Vincendeau et al., 2010). Since the introduction of the highly active antiretroviral therapy, the incidence of severe neurologic disorders such as HIV-1-associated dementia has dropped drastically. However, patients continue to show some CNS dysfunctions known as minor cognitive motor disorder (Gonzalez-Scarano and Martin-Garcia, 2005). More recently, a high prevalence of peripheral neuropathy has been noted in HIV-1-infected individuals as well as a persistence of neurological complications better known as HIV-1-associated neurocognitive disorders (HAND) (Crossley and Brew, 2013). An overall prevalence of HAND in patients with suppressed plasma viraemia was found to reach 52% in the CHAPTER study (Cysique and Brew, 2011). A strong correlation between HIV-1-associated neurocognitive disorders and neuroinflammation in infected individuals has been demonstrated (Adle-Biassette et al., 1999), possibly more so than with the viral load found in the CNS (Gannon et al., 2011). Elevated concentrations of bacterial lipopolysaccharide, cytokines, viral proteins and eicosanoids present in the plasma of infected individuals have been reported to increase the permeability of the blood–brain barrier (BBB) (Strazza et al., 2011; Vincendeau et al., 2010; Wang et al., 2008). Furthermore, in the context of HIV-1 encephalitis, a marked augmentation in matrix metalloproteinases (MMPs) produced by astrocytes can be correlated with the disruption of the BBB (Eugenin et al., 2006; Gramegna et al., 2011). Moreover, certain chemokines produced in the CNS enhance neuroinflammation (such as IP-10, CCL2, MIP-1, CX3CL1 and IL-8) and may promote HIV-1 neuroinvasion by attracting virus-infected leukocytes to this immunologically privileged site (Becker, 2007; Clay et al., 2007; Gesser et al., 1996; Kolb et al., 1999).

Leukotrienes (LTs) are potent proinflammatory lipid mediators that display strong autocrine/paracrine activities and these are produced by innate immune cells when challenged by a pathogenic

* Corresponding author at: Axe des Maladies Infectieuses et Immunitaires, RC709, Centre Hospitalier Universitaire (CHU) de Québec - pavillon CHUL, 2705 boul. Laurier, Québec, Canada G1V 4G2. Tel.: +1 418 525 4444x48274; fax: +1 418 654 2715.
E-mail address: michel.j.tremblay@crchul.ulaval.ca (M.J. Tremblay).

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agent. In stimulated cells, 5-lipoxygenase (5-LO) metabolizes arachidonic acid into either leukotriene B_4 (LTB_4) or cysteinylleukotrienes (cysLTs) which include LTC_4, LTD_4 and LTE_4. Once secreted, LTB_4 can engage BLT1 or BLT2 receptor, whereas cysLTs exert their effect on receptors such as cysLT1, cysLT2 and GPR17 (Haeggstrom and Funk, 2011). Previous studies have reported that LTs along with their cognate receptors are expressed in the brain, notably during HIV-1 infection (Basselin et al., 2011; Froldi et al., 1992; Matsuo et al., 1995).

Astrocytic endfeet are directly in contact with the BBB and contribute in maintaining its integrity and homeostasis (Abbott et al., 2010). Because astrocytes are the most abundant glial cells of the brain and are known to release elevated levels of different soluble factors including cysLTs, it is clear that these cells deserve special attention based on the frequency of HIV-1-mediated neurological complications. We thus assessed whether treatment of astrocytes with LTC_4 can result in production of soluble factors that modulate transmigration of HIV-1-infected primary human $CD4^+$ T cells across a BBB model. We demonstrate here that conditioned medium from LTC_4-treated astrocytes enhances transmigration of HIV-1-infected $CD4^+$ T cells via CX3CL1/fractalkine production and promotes virus transfer to primary human macrophages.

Results

The cysLT 2 receptor is expressed on human astrocytes

We first verified by flow cytometry if human astrocytes express LTB_4 and/or cysLTs receptors. As illustrated in Fig. 1, both the human astrocyticglioma U-87 MG cell line and primary normal human astrocytes (NHA) express high levels of cysLT2. However, no surface expression of the LTB_4 receptor could be detected on these cells (data not shown). Therefore, the following experiments were carried out with LTC_4 because it is considered as a potent cysLT found in the CNS. The less active cysLT metabolite N-Acetyl-LTE_4 (N-Ac-LTE_4), which is 100 times less potent than LTC_4 as a vasoconstricting agent (Foster et al., 1986), was used as a control in our studies.

LTC_4-treated astrocytes produce soluble factors that promote transmigration of HIV-1-infected $CD4^+$ T cells across BBB endothelial cells

The protective immunosurveillance of the CNS is ensured partly by $CD4^+$ T cells. HIV-1 can take advantage of this influx of leukocytes to invade the CNS based on the idea that $CD4^+$ T cells act as a major cellular reservoir for this retrovirus. Therefore, we set out to determine if soluble factors secreted by LTC_4-treated astrocytes could modulate the mechanism of HIV-1 trafficking in the CNS. This goal was reached by performing experiments with the human cerebral microvascular endothelial cell line hCMEC/D3, which closely mimics most of the key characteristics of the BBB (Poller et al., 2008; Weksler et al., 2005). Permeability assays using Blue Dextran showed that the barrier integrity of hCMEC/D3 monolayers grown on collagen-coated transwells (3 μm pore size) was preserved for at least 48 h following confluency (data not shown). Next, we tested the effect of astrocyte-conditioned medium on transmigration of primary human activated $CD4^+$ T cells, infected or not with HIV-1, across this BBB model. To this end, astrocyte-conditioned medium was harvested from U-87 MG astrocytic cells and NHA, which were either treated with LTC_4, N-Ac-LTE_4, or left untreated. The astrocyte-conditioned medium was placed in the lower chamber of the transwells. Finally, $CD4^+$ T cells infected or not with YU2, a brain-derived primary isolate of HIV-1 that utilizes CCR5 as a co-receptor, were placed in the upper chamber and transmigrated cells were quantified 24 h later. Preliminary experiments utilizing supernatants from human astrocytes treated with increasing doses of LTC_4 (i.e. 0.1, 1, 10 and 100 ng/ml) showed a statistically significant induction of $CD4^+$

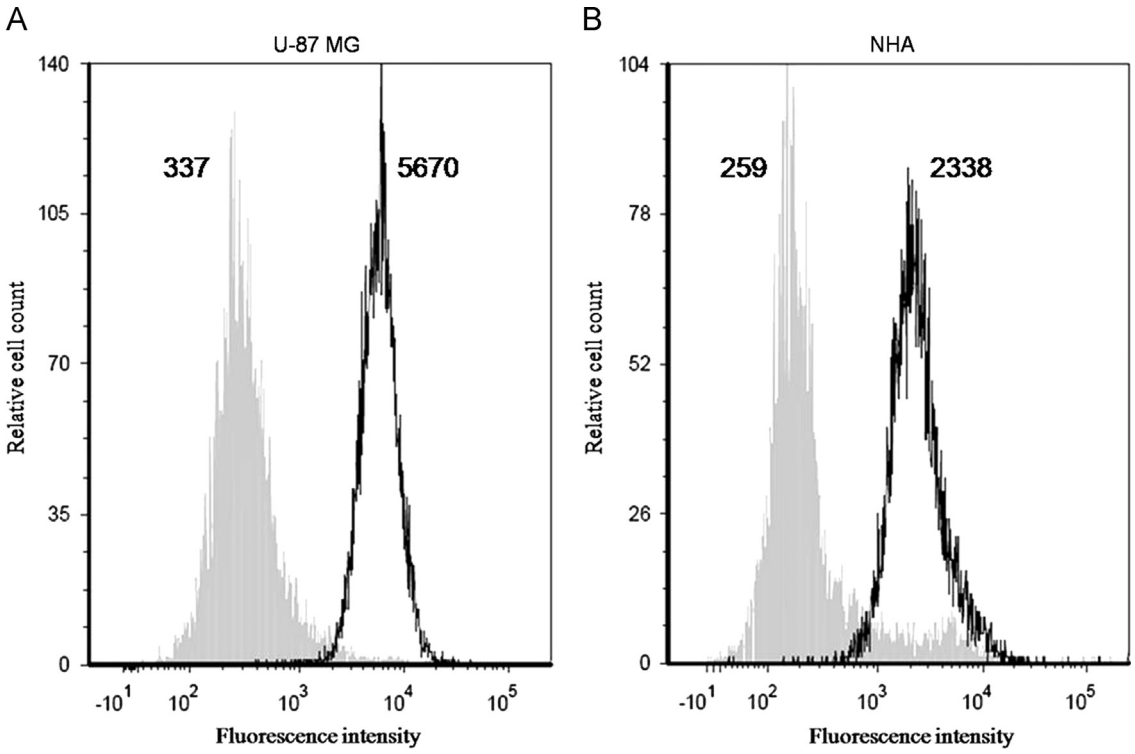

Fig. 1. Human astrocytes express the cysLT2 receptor. Human astrocytoma U-87 MG cells and NHA were incubated either with a rabbit anti-cysLT2 receptor antibody (black line) or a rabbit isotype-matched irrelevant control antibody (gray line) followed by an anti-rabbit antibody conjugated with Alexa Fluor® 488. The results shown are representative of one out of a total of 4 independent experiments.

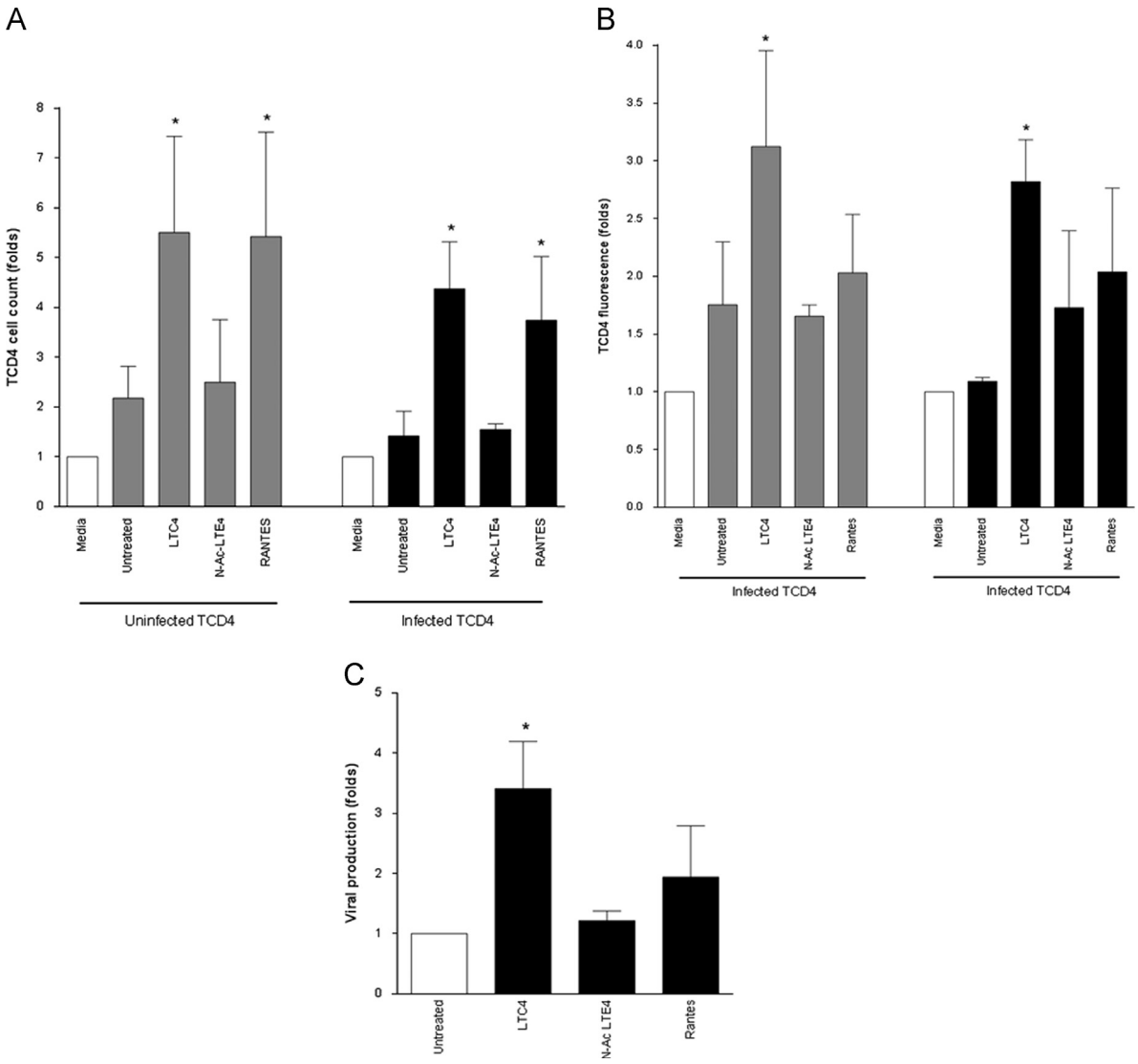

Fig. 2. Conditioned medium from LTC_4-treated astrocytes promotes transmigration of $CD4^+$ T cells across a human BBB model system and enhances subsequent HIV-1 infection of macrophages. First, U-87 MG astrocytic glioma cells (A) and NHA (B) were either left untreated or treated for 24 h with LTC_4, N-Ac-LTE_4. Next, conditioned medium from such untreated and treated astrocytes were placed under the lower chamber of transwells. In some samples, DMEM supplemented with 0.2% BSA (called media/used as a negative controls) and RANTES (used as a positive control for $CD4^+$ T-cell chemotaxis) were introduced directly into the lower chambers. Finally, primary human activated $CD4^+$ T cells either left uninfected or infected with HIV-1 (i.e. YU2) were placed in the upper chamber of transwells and allowed to migrate across a monolayer of hCMEC/D3 human endothelial cells. In studies conducted with NHA (panel B), $CD4^+$ T cells were initially stained with the Calcein AM fluorescent dye prior to their use. Transmigration of $CD4^+$ T cells was estimated either by flow cytometry (A) or fluorometry (B). The means $\pm$ standard deviations (SD) are calculated from 4 independent experiments with triplicate samples (Student *t*-test: *, $p < 0.05$; compared to untreated cells). (C) Primary human MDMs were cocultured for 24 h with HIV-1-infected $CD4^+$ T cells that had transmigrated in response to conditioned medium from U-87 MG cells either left untreated or treated with the listed stimuli. Virus production was monitored 9 days following coculture of MDMs and transmigrated $CD4^+$ T cells by quantifying the major viral core p24 protein, using an in-house ELISA assay. The measured p24 contents for each condition were as follows: 4.0 ± 2.8 ng/mL (untreated), 11.4 ± 6.3 ng/mL (LTC_4), 4.5 ± 2.9 ng/mL (N-Ac-LTE_4), and 5.3 ± 2.1 ng/mL (RANTES). To compensate for donor-to-donor infection variations, the results shown represent the means $\pm$ SD calculated from 3 independent experiments and are expressed as fold increase over media (A and B) or untreated cells (C) (Student *t*-test: *$p < 0.05$; compared to untreated cells).

T cell transmigration at 10 and 100 ng/ml, with a maximal effect at the highest concentration used (data not shown). Therefore, 100 ng/ml of LTC_4 was utilized for the following experiments. As depicted in Fig. 2A, astrocyte-conditioned medium from LTC_4-treated U-87 MG cells induced a marked increase in transmigration of primary human $CD4^+$ T cells compared to media. Cell migration was not promoted when using astrocyte-conditioned medium from U-87 MG cells either left untreated or exposed to the less active analog N-Ac-LTE_4. Interestingly, a comparable enhancement in cell transmigration mediated by conditioned medium from LTC_4-treated U-87 MG cells was observed with both uninfected (left panel) and HIV-1-infected $CD4^+$ T cell cultures (right panel). Initial transmigration studies revealed that conditioned medium from LTC_4-treated NHA was less powerful at promoting migration of $CD4^+$ T cells across hCMEC/D3 endothelial cells (data not shown). Hence, $CD4^+$ T cells were initially stained with the Calcein AM fluorescent dye prior to the transmigration assay in order to more efficiently quantify the lower numbers of transmigrated $CD4^+$ T cells. In agreement with our observations with U-87 MG cells, conditioned medium from LTC_4-treated NHA attracted more $CD4^+$ T cells than conditioned medium from untreated astrocytes (Fig. 2B). Again, treatment of astrocytes with the less active analog N-Ac-LTE_4 failed to induce any significant transmigration of $CD4^+$ T cells across BBB endothelial cells. In all these experiments, the β-chemokine RANTES was used as a positive control while culture medium (i.e. DMEM supplemented with 0.2% BSA) (called media) was used as a negative control for transmigration assays. The use of RANTES in the current work is

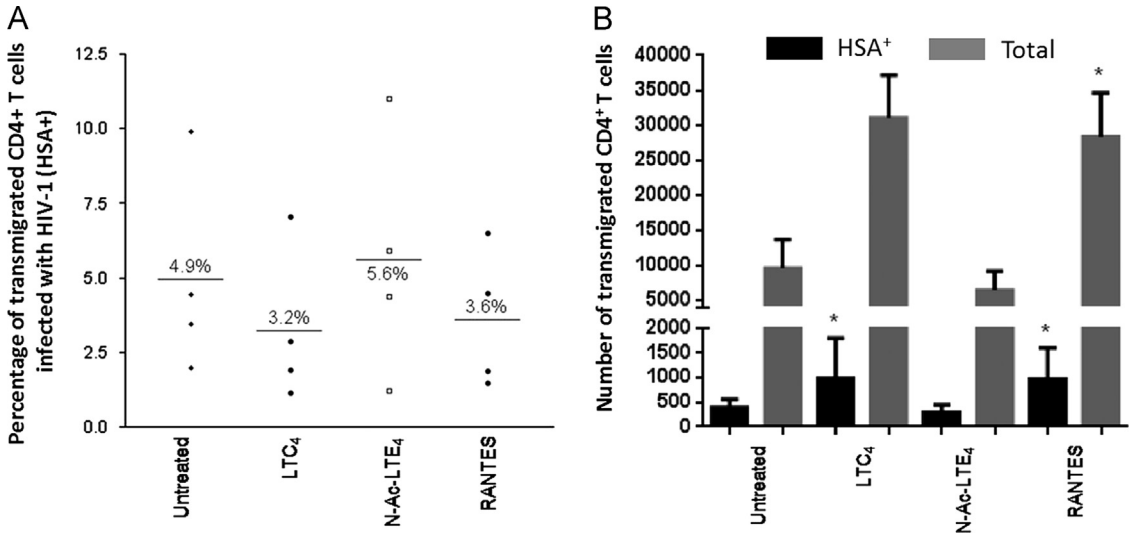

Fig. 3. Conditioned medium from LTC_4-stimulated astrocytes drives transmigration of both uninfected bystander and HIV-1-infected $CD4^+$ T cells. First, U-87 MG cells were either left untreated or treated for 24 h with LTC_4 or N-Ac-LTE_4. Next, conditioned medium from untreated and treated astrocytes were placed under the lower chamber of transwells. In some samples, RANTES was introduced directly into the lower chambers. Finally, primary human activated $CD4^+$ T cells either left uninfected or infected with fully competent reporter HIV-1 particles (i.e. NL4-3Bal-IRES-HSA) for 48 h were placed in the upper chamber of transwells and allowed to migrate across a monolayer of hCMEC/D3 human endothelial cells. After 24 h, transmigrated cells were harvested and stained with the R-Phycoerythrin-conjugated anti-murine HSA/CD24 antibody. Cell transmigration was assessed by flow cytometry by quantifying the percentages of HIV-1-infected $CD4^+$ T cells (i.e. HSA^+) (A) and total amounts of transmigrated $CD4^+$ T cells (i.e. uninfected and virus-infected) (B). Results shown represent the means ± SD calculated from 4 independent experiments and are expressed as number of transmigrated cells (Student *t*-test: $^*p < 0.05$; compared to untreated cells).

founded on the notion that activated $CD4^+$ T cells are known to express CCR5 on their membrane (Monteiro et al., 2011) and also that this chemotactic agent is commonly used in cell transmigration assays (Desmetz et al., 2006; Kawai et al., 1999). Importantly, attraction of $CD4^+$ T cells was not induced by media (DMEM/0.2% BSA) in which LTC_4 was added 24 h prior to the transmigration assay (data not shown), thus demonstrating that the observed augmentation in cell transmigration is not due to LTC_4 by itself but is linked with one or more soluble factor(s) secreted by LTC_4-treated astrocytes.

Perivascular macrophages are the first cells permissive to productive virus infection that circulating leukocytes infected with HIV-1 will meet after crossing the BBB. Therefore, in order to monitor the putative HIV-1 transfer from $CD4^+$ T cells to macrophages, MDMs were cocultured with HIV-1-infected $CD4^+$ T cells that had transmigrated in response to astrocyte-conditioned medium. As shown in Fig. 2C, MDMs produce more progeny virions when cocultured with $CD4^+$ T cells attracted by conditioned medium from LTC_4-treated astrocytes compared to $CD4^+$ T cells that have transmigrated in response to conditioned medium from untreated or N-Ac-LTE_4-treated astrocytes. We next verified if this higher HIV-1 transfer could be explained by a modulatory effect exerted by the astrocyte-conditioned medium from LTC_4-treated cells on the virus infection levels of $CD4^+$ T cells. Although the astrocyte-conditioned medium did increase HIV-1 infection in $CD4^+$ T cells, possibly via some proinflammatory cytokines, viral production levels were the same for target cells exposed to conditioned medium from untreated, LTC_4-, or N-Ac-LTE_4-treated U-87 MG astrocytic cells (data not shown). In parallel, we also tested whether astrocyte-conditioned medium could modulate the infectious potential of YU2 virions through the use of TZM-bl, an indicator cell line highly sensitive to HIV-1 infection. Our data did not reveal any modulation of virus infectivity following exposure of YU2 to conditioned medium from either untreated, LTC_4- or N-Ac-LTE_4-treated U-87 MG cells (data not shown). Therefore, it can be concluded that the enhanced virus production seen in MDMs results from a physical contact with a higher number of HIV-1-infected $CD4^+$ T cells that have transmigrated across the BBB model system in response to conditioned medium from LTC_4-treated U-87 MG astrocytic cells.

A recent work has monitored the HIV-1 infection rate of $CD4^+$ T cells from peripheral blood and it was estimated that 0.06% and 0.14% of circulating $CD4^+$ T cells are infected with HIV-1 during early and chronic infection, respectively (Josefsson et al., 2011). Therefore, we next set out to determine whether cells productively infected with HIV-1 and uninfected bystander cell populations (i.e. Neighboring uninfected cells exposed to soluble factors released by virus-infected cells) were attracted differently by conditioned medium from LTC_4-treated U-87 MG cells. To achieve this goal, activated $CD4^+$ T cells were first infected with fully infectious R5-tropic reporter viruses that encode for a cell surface molecule called the murine heat-stable antigen (HSA/CD24) (used as a reporter molecule). This unique tool permits to discriminate between cells productively infected with HIV-1 (i.e. HSA^+) and uninfected bystander cells (i.e. HSA^-) on the basis of HSA/CD24 surface expression. The mean percentage of activated $CD4^+$ T cells productively infected using this reporter virus was 5.3%, which is in agreement with our previous observations (Imbeault et al., 2009a, 2009b). The percentage of HIV-1-infected $CD4^+$ T cells, as monitored by HSA expression, was lower in the total cell population attracted by conditioned medium from LTC_4-stimulated astrocytes (3.2%) than with conditioned medium from untreated (4.9%) or N-Ac-LTE_4-treated astrocytes (5.6%) (Fig. 3A). A diminished transmigration of $CD4^+$ T cells productively infected with HIV-1 was also seen when using RANTES (3.6%). However, even though the percentage of HIV-1-infected $CD4^+$ T cells is lower in the transmigrated cell population in response to conditioned medium from LTC_4-treated astrocytes, the total number of virus-infected $CD4^+$ T cells (i.e. HSA^+) that have been attracted across our BBB model is still higher when using such astrocyte-conditioned medium (Fig. 3B).

LTC_4 does not induce BBB disruption by increasing MMP-2 or MMP-9 production by astrocytes

It has been reported that astrocytes produce MMPs, which can ultimately disrupt the BBB by degrading tight junction proteins (Gramegna et al., 2011; Ivey et al., 2009). Of these, MMP-2 and

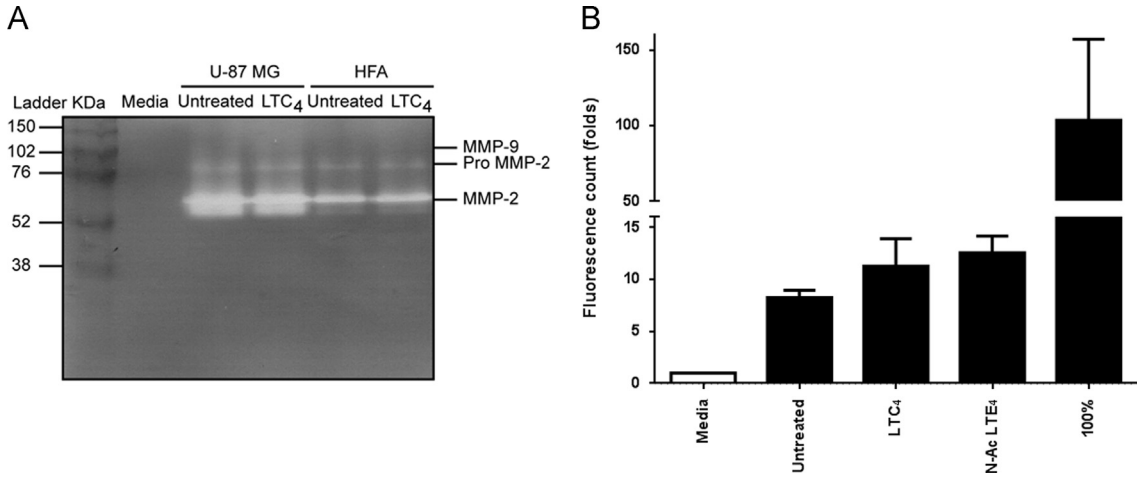

Fig. 4. Conditioned medium from LTC_4-treated astrocytes fails to induce disruption of the BBB: (A) U-87 MG astrocytic glioma cells and NHA were either left untreated or treated for 24 h with LTC_4 or N-Ac-LTE_4. In some samples, cells were treated DMEM supplemented with 0.2% BSA (called media). Next, conditioned media from untreated and treated astrocytes were subjected to an in situ zymography as described in "Materials and methods". Results shown are representative of 3 independent experiments. (B) Dextran-Rhodamine (12.5 μg) was placed over or below (100%) hCMEC/D3 monolayers grown on transwell inserts for 24 h before determining fluorescence intensity (540/625 nm) underneath transwells. Data shown represent the fold increase ± SEM with respect to media calculated from 2 independent experiments.

MMP-9, known to be produced by astrocytes, are often found in the CNS of HIV-1-infected individuals and it was postulated that they might play a key role in BBB disruption (Feng et al., 2011; Qiu et al., 2011; Yang et al., 2007). We thus tested whether LTC_4 treatment induces a deregulation of MMP-2 and MMP-9 secretion by astrocytes. Zymography assays revealed that both U-87 MG cells and NHA produce MMP-2 but not MMP-9 (Fig. 4A). Nonetheless, LTC_4 did not increase MMP-2 secretion by astrocytes nor did it induce any MMP-9 production. In accordance with these results, conditioned medium from LTC_4-stimulated astrocytes failed to significantly increase the passage of a 70-kDa dextran-conjugated fluorescent dye through hCMEC/D3 monolayers (Fig. 4B), suggesting a lack of effect on BBB integrity. To better determine if the more robust transmigration of $CD4^+$ T cells induced by conditioned medium from LTC_4-stimulated astrocytes does not depend on the disruption of the *in vitro* BBB, chemotaxis experiments were performed using collagen-coated transwells but this time in absence of hCMEC/D3 cells. The results obtained from the chemotaxis assays using such transwells showed a similar effect of LTC_4-treated astrocyte conditioned medium, although with a much higher absolute number of transmigrated cells than in the presence of a hCMEC/D3 monolayer (data not shown), which suggest that BBB disruption does not play a significant role in the increased transmigration of $CD4^+$ T cells induced by conditioned medium from LTC_4-treated astrocytes.

CX3CL1/fractalkine is involved in the LTC_4-mediated increase in transmigration of $CD4^+$ T cells

We next set out to shed light on the LTC_4-mediated soluble factor(s) produced by human astrocytes that can drive transmigration of $CD4^+$ T cells across BBB endothelial cells. Since cysLTs have been shown to induce IL-8 production in different cell types (Rola-Pleszczynski and Stankova, 2007) and IL-8 acts as a potent T-cell chemoattractant, we initially tested whether astrocytes could secrete IL-8 upon treatment with LTC_4. In our hands, LTC_4 failed to induce IL-8 secretion by astrocytes as opposed to TNFα and IFNγ that were used as positive controls in this series of investigations (data not shown). Thereafter, mRNA expression of several known chemotactic molecules for $CD4^+$ T cells (i.e. IP-10/CXCL10, MCP-1/CCL2, MCP-2/CCL8, MCP-4/CCL13, MIP-1α/CCL3, MIP-3/CCL20, SLC/CCL21, RANTES/CCL5, PARC/CCL18, TARC/CCL17 and fractalkine/CX3CL1) was measured by quantitative real-time polymerase chain reaction (qRT-PCR) in U-87 MG cells following LTC_4 treatment. Among these chemokines, only fractalkine/CX3CL1 showed a significant increase (4-fold) in mRNA expression in LTC_4-treated U-87 MG cells (Fig. 5A). To directly assess the implication of this chemokine in the increased chemotactic potential of conditioned medium from LTC_4-treated astrocytes, we tested if a CX3CL1/fractalkine blocking antibody could neutralize this phenomenon. Data shown in Fig. 5B indicate that a CX3CL1/fractalkine blocking antibody, but not an isotype-matched irrelevant control antibody, reduces significantly the increased transmigration of HIV-1-infected $CD4^+$ T cells in response to conditioned medium from LTC_4-treated U-87 MG cells (i.e. a reduction of 37.5 ± 15%). The blocking antibody causes a comparable diminution when using conditioned medium from TNFα- and IFNγ-treated U-87 MG cells, which corroborates the importance of CX3CL1/fractalkine in the increased transmigration of $CD4^+$ T cells. To validate further the role of CX3CL1/fractalkine in the more important cell migration across the BBB experimental model system, we achieved gene silencing using RNA interference (RNAi) technology. As depicted in Fig. 6A, CX3CL1/fractalkine mRNA expression is efficiently attenuated in U-87 MG cells following transfection of a pool of four specific small interfering RNAs (siRNAs). Importantly, culture medium from LTC_4-treated astrocytes in which CX3CL1/fractalkine mRNA expression has been reduced could no longer increase the transmigration of HIV-1-infected $CD4^+$ T cells across our BBB model (Fig. 6B). Overall, these results indicate that the LTC_4-dependent secretion of CX3CL1/fractalkine by human astrocytes is responsible for the enhanced transmigration of HIV-1-infected $CD4^+$ T cells through an *in vitro* model of BBB.

Discussion

It has been proposed that HIV-1 can invade the CNS via virus-infected monocytes and $CD4^+$ T cells that cross the BBB (Fischer-Smith et al., 2001; Glass et al., 1995; Haas et al., 2000, 2003; Harrington et al., 2005; Persidsky et al., 1999; Schnell et al., 2011; Wang et al., 2008). Until now, most studies have focused on the neuroinvasion of HIV-1 via monocytes. Nevertheless, the putative mechanism(s) by which HIV-1-infected $CD4^+$ T cells are attracted in the CNS remains to be more fully elucidated. It has been shown that the cysLT2 receptor is found in the CNS under pathological conditions, often on astrocytes (Heise et al., 2000; Huang et al.,

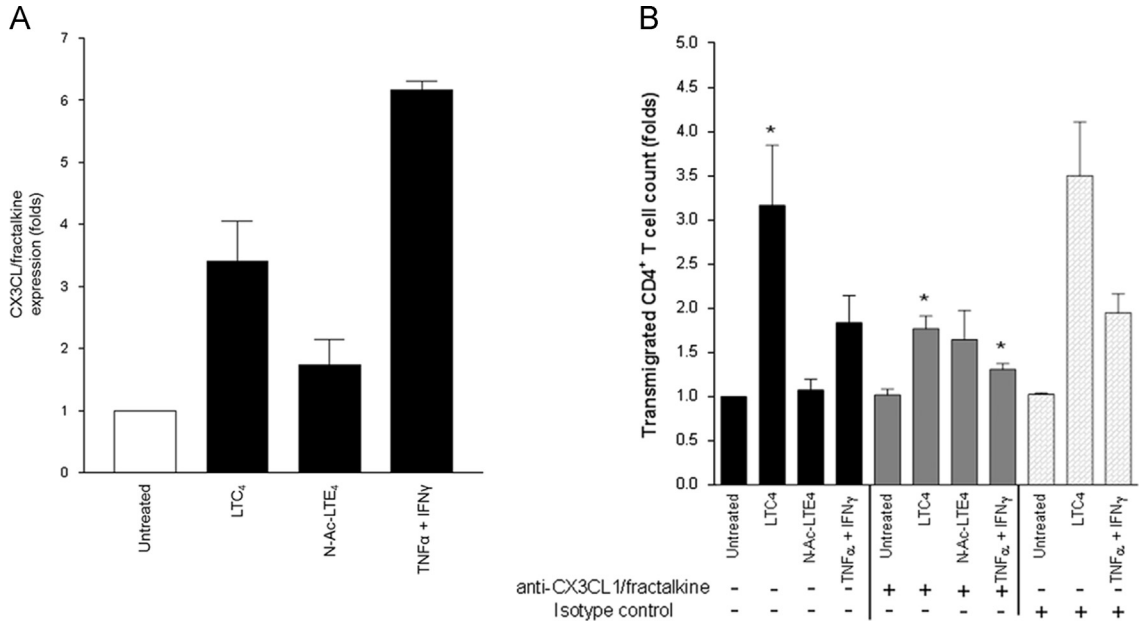

Fig. 5. Higher transmigration of HIV-1-infected CD4$^+$ T cells in response to conditioned medium from LTC_4-treated astrocytes relies on CX3CL1/fractalkine: (A) U-87 MG cells were either left untreated or treated for 4 h with LTC_4, N-Ac-LTE_4, or a combination of TNFα and IFNγ before quantifying CX3CL1/fractalkine mRNA by qRT-PCR. Results shown represent the fold increase ± SD with respect to untreated cells calculated from 3 independent experiments. (B) U-87 MG cells were initially either left untreated or treated for 24 h with LTC_4, N-Ac-LTE_4, or a combination of TNFα and IFNγ. Next, conditioned medium from untreated and treated astrocytes was incubated for 2 h at 37 °C either with a neutralizing anti-CX3CL1/fractalkine antibody or an isotype-matched irrelevant antibody and next placed under the lower chamber of transwells. Finally, primary human CD4$^+$ T cells infected with HIV-1 (i.e. YU2) were placed in the upper chamber of transwells and allowed to migrate across a monolayer of hCMEC/D3 human endothelial cells. The number or transmigrated cells was determined by flow cytometry. The results shown represent the mean number of transmigrated cells ± SD calculated from 3 independent experiments with different PMBC donors and are expressed in folds over untreated cells (Student *t*-test: $*p < 0.05$; compared to untreated cells).

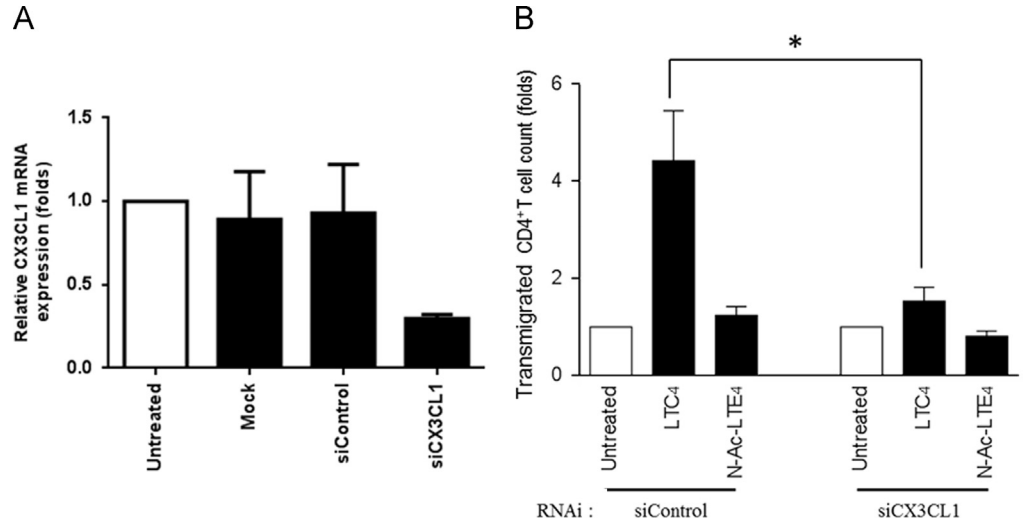

Fig. 6. Silencing of CX3CL1/fractalkine in human astrocytes abrogates the LTC_4-induced higher transmigration of HIV-1-infected CD4$^+$ T cells: (A) U-87 MG cells were either left untreated or transfected with CX3CL1/fractalkine-specific siRNAs (siCX3CL1) or a nontargeting control siRNA (siControl). Thereafter, CX3CL1/fractalkine mRNA levels in transfected cells were quantified by qRT-PCR at 3 days following siRNA transfection. (B) Transfected cells were either left untreated or treated for 24 h with LTC_4 or N-Ac-LTE_4. Next, conditioned media were then harvested and used in a transmigration assay with HIV-1-infected CD4$^+$ T cells. Transmigrated cells were counted by flow cytometry. The results shown represent the mean number of transmigrated cells ± SD calculated from 3 independent experiments with different PMBC donors and are expressed in folds with respect to untreated cells (Student *t*-test: $*p < 0.05$: compared to untreated cells).

2008; Qi et al., 2011; Weksler et al., 2005; Zhao et al., 2011). In corollary, our results indicate that the cysLT2 receptor is highly expressed on human astrocytes (both a continuous cell line/U-87 MG and primary cells/NHA). As for cysLTs, they have been detected in the cerebrospinal fluid (CSF) of HIV-1-infected individuals (Froldi et al., 1992, 1995; Genis et al., 1992), and they are also present in infected brain tissues or virus-infected CNS cell types (Basselin et al., 2011; Froldi et al., 1992, 1995; Genis et al., 1992; Maccarrone et al., 2000).

We investigated whether treatment of human astrocytes by cysLTs could mediate production of soluble factor(s) that can in turn affect transmigration of HIV-1-infected CD4$^+$ T cells across an *in vitro* BBB model. Along with LTD_4, LTC_4 is known to be one of the most potent cysLTs. Therefore, we used only LTC_4 to stimulate astrocytes since all cysLTs engage the same receptors. We report here that conditioned medium from LTC_4-treated astrocytes induces a more potent transmigration of HIV-1-infected CD4$^+$ T cells across BBB endothelial cells than supernatants from

untreated astrocytes. The LTC_4-mediated enhancement in cell transmigration was seen when using a final concentration of LTC_4 (i.e. 100 ng/ml) that is somewhat higher than what is measured in brains of HIV-1 transgenic rats or found in CSF obtained from HIV-1-infected individuals (Basselin et al., 2011; Froldi et al., 1992, 1995). However, it should be mentioned that the microenvironment of astroglial cells in inflamed rodent brains may not correlate with what is produced by astrocytes in humans carrying HIV-1. Moreover, levels of LTs in CSF are probably an underestimation of the amount produced in brain tissue. Indeed, because LTs can be secreted locally by glial cells, concentrations similar or higher to those used in our work are likely reached in the microenvironment surrounding brain microglial cells based on the notion that pg of LTs are measured per g of brain tissue.

In a search for the molecule(s) responsible for this increased cell transmigration, we found that LTC_4-treated human astrocytes produce up to 4-fold more CX3CL1/fractalkine mRNA than untreated astrocytes. The biological implication of this chemokine in the enhanced transmigration of HIV-1-infected $CD4^+$ T cells was further confirmed using a neutralizing antibody and siRNA-based gene silencing. Additional experiments have been performed with primary astrocytes (i.e. NHA) in an attempt to validate conclusions made with the human astrocyticglioma cell line U-87 MG. Unfortunately, we were unable to detect a statistically significant difference in CX3CL1/fractalkine mRNA expression (as assessed by qRT-PCR) between untreated and LTC_4-treated NHA (data not shown). This might be related to the previously reported fibroblast contamination seen in early passage cultures prepared from dissociated CNS tissue (McCarthy et al., 1995). Indeed, fibroblast contamination is a widespread phenomenon when culturing primary human cells since fibroblasts display faster generation times and can over populate more highly differentiated cells *in vitro* (Pal and Grover, 1983). The possibility that a fibroblast contamination in NHA might act as a confounding factor is fully supported by the demonstrated capacity of LTC_4 to modulate some fibroblast functions (Medina et al., 1994; Phan et al., 1988), which in turn could affect the biology of astrocytes. Moreover, it has been reported that fibroblast cells can express CX3CL1/fractalkine under different experimental conditions (Husberg et al., 2008; Isozaki et al., 2011; Jones et al., 2013; Ohta et al., 2010).

As reviewed previously, CX3CL1/fractalkine has been proposed to play a pivotal role in spreading of HIV-1 infection in the organism via virus-infected lymphocytes (Becker, 2007; Cotter et al., 2002). Our data suggest that CX3CL1/fractalkine production by astrocytes induced by the cysLTs LTC_4 may also play an important role in the HIV-1 neuroinvasion. Nevertheless, chemotaxis of $CD4^+$ T cells is known to be induced by many other chemokines, lipid mediators and interleukins. Additional studies are needed to determine whether another yet-to-be defined soluble factor is produced by LTC_4-treated astrocytes which may also promote transmigration of HIV-1-infected $CD4^+$ T cells across BBB endothelial cells.

Since activated $CD4^+$ T cells are known to be highly permissive to HIV-1 infection, attraction of this cell population in the CNS by LTC_4-stimulated astrocytes may provide a renewed source of target cells thus allowing a sustained viral replication in this body compartment. We also provide evidence that the increased numbers of $CD4^+$ T cells attracted across the BBB give rise to a more potent infection *in trans* of macrophages, which constitute a stable viral reservoir that facilitate spread of HIV-1. Therefore, it can be speculated that stimulation of human astrocytes with cysLTs such as LTC_4 induces neuroinvasion and a sustained viral replication in the CNS via a possible HIV-1 transfer to perivascular macrophages.

It is of interest to note that our data suggest also that conditioned medium from LTC_4-treated astrocytes attracts both uninfected bystander cells and $CD4^+$ T cells productively infected with HIV-1 with a slightly higher efficiency for the former cell population. It is possible that a fraction of HIV-1-infected $CD4^+$ T cells become less responsive to subsequent stimulation by the chemotactic molecule CX3CL1/fractalkine, or yet again found to be in an anergic state rendering them unable to efficiently transmigrate (Wherry, 2011). It has been reported previously that HIV-1 infection of leukocytes induces a better transmigration across the BBB in response to the chemokine CCL2, due to an increased expression of CCR2 (Eugenin et al., 2006). These data suggest that such virus-infected cells do not lose their inherent ability to transmigrate at least in response to CCL2. It is possible that HIV-1-infected $CD4^+$ T cells do not respond as well to CX3CL1/fractalkine. The explanation for this discrepancy is currently unknown but it should be stated that the experimental procedure performed by Eugenin et al. (2006) does not permit to discriminate between migration of uninfected bystander cells versus HIV-1-infected leukocytes. Nonetheless, flow cytometric analyses of the CX3CL1/fractalkine receptor CX3CR1 indicate that it is expressed on $CD4^+$ T cells that transmigrate in our assay. However, its surface expression level is comparable in transmigrated HIV-1-infected $CD4^+$ T cells upon exposure to culture medium from human astrocytes either left untreated or treated with LTC_4 or N-Ac-LTE_4 (data not shown). It can be concluded that CX3CL1/fractalkine does not seem to modulate its cell surface receptor CX3CR1 level at least in transmigrating HIV-1-infected $CD4^+$ T cells.

Furthermore, we found that MMP-2 but not MMP-9 is produced by human astrocytes, and that LTC_4 does not stimulate secretion of MMP-2. In addition, LTC_4 treatment does not induce any alteration of the barrier integrity of hCMEC/D3 monolayer. The neuroinvasion of HIV-1 via $CD4^+$ T cells in response to conditioned medium from LTC_4-stimulated astrocytes is thus independent of a BBB disruption caused by gelatin-binding MMPs. Several studies have suggested that MMP activity is an important mediator involved in the BBB disruption seen in HIV-1-carrying patients showing neurological disorders, and HIV-1 particles and viral proteins (e.g. Tat and gp120) were shown to induce expression of MMP-2 and MMP-9 in astrocytes (Ivey et al., 2009; Louboutin et al., 2010, 2011). We can thus propose a model for HIV-1-related neuropathology in which physical damages to the BBB are mostly caused by the virus itself, whereas cysLTs such as LTC_4 participate to the neuroinvasion process by inducing the secretion of CX3CL1/fractalkine and other chemotactic molecules by astrocytes.

Of course, further investigations will be needed to establish the possible implications of the astrocyte secretome induced by LTC_4 on a more physiological BBB model which would be also sheeted by perivascular macrophages and pericytes. Indeed, infection of astrocytes by HIV-1 was shown to compromise BBB integrity through endothelial apoptosis, misguided astrocyte endfeet and dysregulation of lipoxygenase/cyclooxygenase (Eugenin et al., 2011). Analyzing the effect of LTC_4 on the astrocyte–BBB relationship in the context of HIV-1 infection would shed light on the more precise mechanism(s) governing neuroinvasion and neuropathology processes.

Conclusions

Our results underline the complex interplay between neuroinvasion and neuroinflammation in the context of HIV-1 infection. The sustained prevalence of neurological complications that arise in virus-infected individuals is intimately linked to the neuroinflammation processes induced by HIV-1. Eicosanoids (such as LTs and prostaglandins) – which are normally produced under

pathological conditions – are known to negatively modulate HIV-1 infection in different cell types including some of which are found in the CNS (Bertin et al., 2012). On the other hand, we show herein that cysLTs, produced in the CNS of infected individuals, can activate astrocytes and promote the neuroinvasion of HIV-1. *In vivo* experiments with non-human primates should be considered to better understand the overall contribution of LTs in the neuropathogenesis of HIV-1 infection.

Materials and methods

Antibodies and reagents

LTC_4 and its less active metabolite N-Acetyl-LTE_4 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Surface expression of LT receptors was determined by flow cytometry using either a rabbit anti-cysLT2 antibody (Cayman Chemical) followed by a goat anti-rabbit antibody conjugated with Alexa Fluor® 488 (Molecular Probes, Burlington, Ont., Canada) or a biotinylated anti-BLT1 antibody (Cayman Chemical) followed by a streptavidin-R-phycoerythrin (BD Bioscience, Mississauga, Ont., Canada). Productive HIV-1 infection of primary human $CD4^+$ T cells with fully competent HSA-encoding reporter virus (see below for more details) was assessed by flow cytometry using the R-phycoerythrin conjugated anti-HSA/CD24 antibody obtained from BD Bioscience (Mississauga, Ont., Canada). The R-phycoerythrin conjugated anti-CX3CR1 and its isotype-matched control antibody were purchased from Biolegends (San Diego, CA, USA). The anti-CX3CL1/fractalkine blocking antibody was purchased from R&D Systems (Minneapolis, MN, USA). IL-8 was quantified using the BD Biosciences Human IL-8 ELISA Set. High glucose DMEM and RPMI-1640 media were purchased from Wisent Bioproducts (Montréal, Que., Canada). Macrophage colony-stimulating factor (M-CSF) was purchased from GenScript Corporation (Piscataway, NJ, USA). Primers for PCR were purchased from Integrated DNA Technologies (San Diego, CA, USA). ThinCerts transwells (3.0 μm pore size) were purchased from Greiner Bio-One (Frickenhausen, Baden-Württemberg, Germany). Purified IFNγ and TNFα were obtained from Biolegend (San Diego, CA, USA). Bovine serum albumin (BSA) (20% sterile and filtered solution) was purchased from Wisent Bioproducts. All culture flaks and plates were purchased from BD Biosciences. Collagen and Blue Dextran (MW: 70 kDa) were purchased from Sigma Aldrich (St. Louis, MO, USA). Calcein AM was obtained from Invitrogen (Burlington, Ont., Canada), lithium chloride (LiCl) was purchased from EMD chemicals (Gibbstown, NJ, USA), whereas Dextran-Rhodamine fluorescent dye (70 kDa) and Lipofectamine™ RNAiMAX transfection reagent were purchased from Life Technologies (Carlsbad, CA, USA).

Plasmids

pYU2 is a macrophage/CCR5-tropic HIV-1 molecular clone isolated directly from human brain (obtained through the NIH AIDS Research and Reference Reagent Program, Germantown, MD,USA) (Li et al., 1991). The infectious molecular clone pNL4-3Bal-IRES-HSA was recently described (Imbeault et al., 2009a). Briefly, besides encoding all HIV-1 proteins and producing R5 (Bal)-tropic virions, this NL4-3-based vector additionally codes for the cell surface murine heat-stable antigen (HSA)/CD24, enabling for efficient early identification of cells productively infected with HIV-1.

Cell culture

The human glioblastoma-astrocytoma, epithelial-like cell line U-87 MG was obtained from the American Type Culture Collection (ATCC HTB-14, Manassas, VA, USA). NHA were purchased from Lonza (Walkersville, MD, USA). In order to obtain astrocyte-conditioned medium (additional details can be found in following section entitled "Transmigration assay"), NHA were cultured in astrocyte basal medium supplemented with astrocyte growth medium (Lonza). Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by Ficoll-Hypaque gradient (Wisent Inc., St-Bruno, QC, Canada). Next, PBMCs were plated at a final concentration of 1×10^7 cells/ml in 75 cm^2 flasks (BD Biosciences) for 2 h in order to separate by adherence monocytes from the other non-adherent cells. After washing with endotoxin-free phosphate-buffered saline (PBS) (Sigma-Aldrich), monocytes were cultured in complete RPMI-1640 medium (that is RPMI-1640 supplemented with 5% heat-inactivated autologous serum, 100 U/ml penicillin G and 100 μg/ml streptomycin) in the presence of M-CSF (10 ng/ml) for 6 days to obtain primary human monocyte-derived macrophages (MDMs). Cells were then recovered with a soft cell scraper and plated in 24-well plates at a final concentration of 1×10^5 cells per well in RPMI-1640 medium supplemented with 5% autologous serum. Non-adherent cells obtained from the same donors were used to isolate $CD4^+$ T cells by a negative selection process using the EasySep $CD4^+$ T cell enrichment kit (Vancouver, BC, Canada). Cells were then activated with the mitogenic agent PHA-L (1 μg/ml) for 3 days prior their use and maintained in complete RPMI-1640 culture medium supplemented with recombinant human IL-2 (30 U/ml) at a density of 2×10^6 cells/ml. Human embryonic kidney (HEK) 293T (ATCC) and TZM-bl cells (NIH AIDS Research and Reference Reagent Program) were cultured in complete Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. The immortalized human cerebral microvascular endothelial cell line hCMEC/D3, which has been shown to constitutively express many endothelial and/or BBB markers with the appropriate subcellular localization pattern, was maintained in culture as described previously (Poller et al., 2008; Weksler et al., 2005). The hCMEC/D3 cell line was a generous gift from Dr. Couraud (Institut Cochin, France) under the license from the Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France).

Virus production

Viruses were produced by the calcium phosphate coprecipitation method in 293T cells as described previously (Fortin et al., 1997). In brief, 293T cells were transiently transfected with pYU2 and pNL4-3Bal-IRES-HSA to produce fully infectious R5-tropic HIV-1 particles and fully competent HSA-encoding reporter virions, respectively. Virus preparations were normalized for virion content by using an in-house enzymatic assay specific for the major viral p24 protein. In this test, two distinct monoclonal anti-p24 antibodies (i.e. 183-H12-5C and 31-90-25) are used in combination to quantify p24 levels (Bounou et al., 2002). In addition, the infectivity of our virus stocks was assessed using TZM-bl indicator cells. This cell line is a genetically modified HeLa derived cell line expressing large amounts of cell surface CD4, CCR5 and CXCR4 (Steckbeck et al., 2005). These cells carry separate integrated copies of the luciferase and β-galactosidase genes under the control of the HIV-1promoter and are highly susceptible to infection with different variants (both R5- and X4-tropic). Virus preparations that did not allow sufficient reporter gene activity were discarded.

HIV-1 infection

PHA-stimulated primary human $CD4^+$ T cells were inoculated with a fixed amount of YU2 virus (i.e. 10 ng of p24 per 1×10^5 cells). Two days following HIV-1 infection, cells were

thoroughly washed and resuspended in DMEM medium supplemented with 0.2% BSA for transmigrations assays. In some experiments, virus-infected $CD4^+$ T cells that had transmigrated across our BBB model were thoroughly washed with HBSS 1X and then cocultured with MDMs for 24 h. Thereafter, MDMs were thoroughly washed three times with HBSS 1X and incubated in complete RPMI-1640 medium. No contaminated $CD4^+$ T cells could be observed by microscopy (data not shown). Cell-free supernatants from MDMs were harvested 9 days post-infection to quantify *trans* infection levels. Virus production was estimated by measuring p24 levels in cell-free culture supernatants by ELISA.

Flow cytometry analysis

Before staining, cells were incubated for 15 min at 4 °C in PBS containing 0.1% sodium azide, 10% heat-inactivated human fibrin-depleted plasma, 10% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 10% FBS to block non-specific binding sites and washed once with PBS containing 0.1% sodium azide and supplemented with 5% FBS. To monitor cell surface expression of LT receptors and CX3CR1, cells were incubated with specific antibodies or with an appropriate isotype-matched irrelevant control antibody (for non-specific staining) for 30 min at 4 °C. Cells were then washed with PBS supplemented with 0.1% sodium azide and 5% FBS and fixed in a 2% paraformaldehyde solution before analysis by flow cytometry (Epics ELITE ESP; Coulter Electronics).

Quantitative real-time polymerase chain reaction

For detection and quantification of CX3CL1/fractalkine mRNA (sense: GCAGCCTCACAGTCCTTACC; antisense: GTCTCTGCTCTGCCCATTTC), cells were either left untreated or treated for 4 h before extracting total RNA which was purified utilizing the illustraRNAspin RNA isolation kit (GE Healthcare life sciences, Piscataway, NJ, USA). cDNAs were generated with the Moloney murine leukemia virus (M-MLV) reverse transcriptase and random primers as indicated by the manufacturer (Invitrogen). Amplification of cDNA was achieved using the SYBR Green PCR Master Mix (Life Technologies). To ensure quantification precision, all real-time PCR amplified samples were normalized using the 18S housekeeping gene (sense: TAGAGGGACAAGTGGCGTTC; antisense: CGCTGAGCCAGTCAGTGT). The cycling conditions used for the Applied Biosystems 7500 sequence detection system included a hot start (50 °C for 2 min and 95 °C for 10 min), followed by 40 cycles of denaturation (95 °C for 15 s) and extension (63 °C for 1 min) with end point acquisition.

Zymography assay

Gelatin zymography was performed as described previously with slight modifications (Hu and Beeton, 2010). In brief, U-87 MG astrocytic cells and NHA were cultured in 6 well plates (3.5×10^5 cells per well) for 24 h. Next, cells were either left untreated or treated with LTC_4 or N-Ac-LTE_4 (100 ng/ml). Forty-eight hours post-stimulation, culture medium for astrocytes (mock) or filtered astrocyte-conditioned medium was mixed with an equal volume of 2× zymography buffer (125 mM Tris hydrochloride [pH 6.8], 4% sodium dodecyl sulfate (SDS), 20% glycerol and 0.25% bromophenol blue). Then, samples were electrophoresed on a 10% (w/v) polyacrylamide gel containing 0.1% (w/v) gelatin (MP Biomedicals, Solon, OH, USA). After electrophoresis, the gel was washed for 30 min with 1× renaturating buffer (Novex® Zymogram from Invitrogen, Burlington, Ont., Canada) followed by 30 min incubation with 1× developing buffer (Novex® Zymogram) with gentle shaking. After an overnight incubation with a fresh solution of developing buffer at 37 °C, gels were rinsed, stained for 1 h in Coomassie Brilliant Blue solution (Bio-Rad laboratories, Mississauga, Ont., Canada), and then destained in a 10% acetic acid and 30% methanol solution.

Transmigration assay

First, hCMEC/D3 cells were cultured in collagen-coated ThinCerts transwells (12×10^3 per transwell) for 7 days in complete EBM-2 culture medium in presence of 10 mM LiCl. To obtain astrocyte-conditioned media, U-87 MG astrocytic cells or NHA were plated in 6 well plates (3.5×10^5 per well) in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin. One day after, cells were washed and stimulated with LTC_4 or N-Ac-LTE_4 (100 ng/ml) in DMEM/0.2% BSA or left untreated for 24 h, before harvesting cell-free supernatants. PHA-activated primary human $CD4^+$ T cells either left uninfected or infected with HIV-1 were resuspended in DMEM/0.2% BSA at a density of 20×10^6 cells/ml. Next, cells were overlayed on transwells (5×10^5 per transwell). Alternatively, $CD4^+$ T cells were stained with the Calcein AM fluorescent dye (10 μM) for 30 min and then thoroughly washed before being resuspended prior to transmigration. Thereafter, filtered astrocyte-conditioned medium (0.65 ml) was placed in the lower chamber. After 24 h, transmigrated $CD4^+$ T cells were either counted by flow cytometry or quantified by fluorometry at 460/520 nm (MLX microtiter luminometer, Dynex Technologies, Chantilly, VA, USA).

siRNA silencing of CX3CL1/fractalkine

U-87 MG were plated 24 h before transfection in 6 well plates (3×10^5 cells/well) in 2.5 ml of DMEM medium supplemented with 10% heat-inactivated FBS without antibiotics. Cells were transfected with 10 pmoles of CX3CL1/fractalkine-specific siRNA (siGENOMESMARTpool, Thermoscientific Dharmacon) or nontargeting siRNA (AllStars Negative Control, Qiagen) using Lipofectamine RNAiMAX as indicated by the manufacturer (Invitrogen). Cells were washed at 24 h after transfection and cultured in fresh media (DMEM/10% FBS without antibiotics). Forty-height hours after transfection, astrocytes were either left untreated or stimulated with 100 ng/ml of LTC_4 or N-Ac-LTE_4 for 24 h. Cells were then lysed for CX3CL1/fractalkine mRNA quantification and supernatants were utilized for transmigration experiments.

Statistical analysis

All experiments were repeated at least three times with different donors for $CD4^+$ T cells and MDMs, and each figure combines the results obtained with all the different donors unless otherwise specified. The statistical significance of the difference between groups was determined using the Student *t*-test. Calculations were made with Prism version 3.03 (GraphPad Software Inc., La Jolla, CA, USA). The *P* values < 0.05 were considered statistically significant.

Authors' contributions

J.B. carried out most studies depicted in the current work, performed the statistical analysis and drafted the manuscript. P.J., C. B. and M.A.R. participated to some experiments described in the study. M.J.T. participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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